ZAP PROTEIN AND RELATED COMPOSITIONS AND METHODS

5   This invention was made with support under United States
Government Public Health Service Grant CA 30488.
Accordingly, the United States government has certain
rights in this invention.

10 Throughout this application, various publications are.
referenced. Full citations for these publications may be
found immediately preceding the claims. The disclosures
of these publications are hereby incorporated by
reference into this application in order to more fully
15 describe the state of the art as of the date of the
invention described and claimed herein.

Background of the Invention

20 Vertebrate cells have evolved a number of defense
mechanisms to prevent or inhibit viral replication after
an infection. A remarkable array of such antiviral
proteins are induced by interferon (2), including: PKR, a
double-stranded RNA-dependent kinase that phosphorylates
25 eIF-2α and shuts down translation (3); the Mx proteins,
GTPasses that block viral gene expression by unknown
mechanismsm (4); and oligoA synthetases (5), producing
2′, 5′-oligodenylates (6) that activate Rnase L to
degrade both mRNAs. In some cases the antiviral state
30 involves a drastic shutoff of host functions. In other
cases, there is a more specific block to viral
replication or gene expression. While many parallel
pathways have been uncovered, it is likely that still
more antiviral proteins remain to be found.

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Summary of the Invention

This invention provides an isolated ZAP protein.

5   This invention further provides an isolated nucleic acid
which encodes a ZAP protein.

This invention further provides an expression vector
comprising a nucleic acid sequence encoding a ZAP
10 protein.

This invention further provides a method for increasing
the amount of ZAP protein in a mammalian cell which
comprises contacting the cell with a ZAP protein under
15 conditions permitting entry of the ZAP protein into the
cell, so as to thereby increase the amount of ZAP protein
in the mammalian cell.

This invention further provides a method for increasing
20 the expression of ZAP protein in a mammalian cell which
comprises introducing into the cell an expression vector
comprising a nucleic acid sequence encoding a ZAP
protein, so as to thereby increase ZAP protein expression
in the mammalian cell.
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This invention further provides a method for increasing
resistance to a virus in a mammalian cell which comprises
contacting the cell with a ZAP protein specific for that
virus under conditions permitting entry of the ZAP
30 protein into the cell, so as to thereby increase
resistance to the virus in the cell.

This invention further provides a method for increasing
resistance to a virus in a mammalian cell which comprises

introducing into the cell an expression vector comprising
a nucleic acid sequence encoding a ZAP protein specific
for that virus, so as to thereby increase resistance to
the virus in the mammalian cell.

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This invention further provides a method for increasing
the amount of ZAP protein in a subject's cells which
comprises administering to the subject an amount of ZAP
protein effective to increase the amount of ZAP protein

10 in the subject's cells.

This invention further provides a method for increasing
resistance to a virus in a subject which comprises
administering to the subject an amount of ZAP protein

15 specific for that virus effective to increase the amount
of ZAP protein in the subject's cells, so as to thereby
increase resistance to the virus in the subject.

This invention further provides a method for determining

20 whether an agent increases ZAP protein expression in a
mammalian cell which comprises: (a) contacting the cell
with the agent under conditions permitting ZAP protein
expression; (b) determining the resulting amount of ZAP
protein expression in the cell; and (c) comparing the

25 amount of expression determined in step (b) with the
amount of ZAP protein expression determined in the
absence of the agent, whereby a greater amount of ZAP
protein expression in the presence of the agent relative
to that in the absence of the agent indicates that the

30 agent increases ZAP protein expression in a mammalian
cell.

This invention further provides a method for determining
whether an agent increases resistance to a virus in a

mammalian cell, which comprises: (a) contacting the agent
with a mammalian cell having introduced thereto an
expression vector comprising a nucleic acid sequence
corresponding to the virus operatively linked to a
5   reporter sequence whose expression in a mammalian cell
gives rise to a detectable signal, wherein RNA
corresponding to the virus is known to be degraded by a
ZAP protein present in the cell; (b) determining the
amount of signal produced in the cell by the reporter
10   sequence after contact with the agent; and (c) comparing
the amount of signal determined in step (b) to that
produced in the absence of the agent, whereby the amount
of signal produced in the presence of the agent being
less than that produced in the absence of the agent
15   indicates that the agent increases resistance to the
virus in the cell.

This invention further provides a composition comprising
a ZAP protein and a pharmaceutically acceptable carrier.
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This invention further provides a composition comprising
an expression vector comprising a nucleic acid sequence
encoding a ZAP protein, and a pharmaceutically acceptable
carrier.
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This invention further provides an article of manufacture
comprising a packaging material having therein a ZAP
protein and a label indicating a use for the ZAP protein
for increasing resistance to a virus in a subject.
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Finally, this invention provides an article of
manufacture comprising a packaging material having
therein an expression vector comprising a nucleic acid
sequence encoding a ZAP protein, and a label indicating a

use for the expression vector for increasing resistance
to a virus in a subject.

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Brief Description of the Figures

Figure 1 Schematic representation of pBabe-HAZ construct, MLV viral RNA packaging signal; HA, hemagglutinin epitope tag; EcoRI-NotI, linker sequence containing EcoRI and NotI sites; LoxP, LoxP as sequence for recognition by Cre recombinase.

Figure 2A Resistance of NZAP-zeo expressing cells to virus infection. Cre recombinase was stably introduced into 11D3 cells by cotransformation with pGk-puro. Five puromycin-resistant clones were expanded and tested for resistance to Eco-Luc virus (lower panel). Deletion of NZAP-zeo DNA in each cell line was monitored by PCR (upper panel).

Figure 2B The NZAP-zeo fragment was recovered and reintroduced into naïve Rat2 cells. These cells were compared with the original L1D3 cells for the resistance to Eco-Luc virus. Rat2, wild type Rat2 cells; L1D3 cells isolated from the screening; Rat2-HAZ, Rat2 cells expressing pBabe-HAZ empty vector; Rat2-NZAP-zeo, Rat 2 cells expressing recovered pBabe-Nzap-zeo.

Figure 2C Cells were infected with wild-type MuLV at low multiplicity. The culture supernatants were harvested 2, 4, 6, 8 and 10 days after infection and were analyzed for reverse transcriptase (RT) activity to measure the spread of the virus. RT signals were quantified by Phospoimager and plotted.

Figure 3A Schematic representation of rZAP and related sequences. The cDNA fragment recovered from L1D3 cells.

ATG, start codon; TAG stop codon in 5'UTR; NZAP-zeo, open reading frame of rZAP and Zeo fusion protein.

Figure 3B Blast search of Genbank database with NZAP
5   fragment identified two mouse EST clones (mEST995 and mEST896) that have high sequence similarity to rZAP. These two clones were obtained from ATTC and sequences. The 3' end sequence of mEST995 was used to design a primer to PCR amplify full-length rZAP from Rat2 cDNA
10 library.   Deduced amino acid sequences are compared schematically. The numbers of amino acids of each coding sequence are indicated.   The positions of four CCCH finger motifs are indicated by black boxes and the sequences of the motifs in rZAP are shown.
15

Figure 3C   Northern blot of tissue mRNAs of rat probed with rZAP cDNA.

Figure 4A Analysis of the position of the block to virus
20 infection.   Cells were infected with either Eco-Luc virus (upper panel) or Eco-GFP virus (lower panel) at various dilutions.   Viral DNA was extracted 24 h post-infection and detected by using PCR using primers that specifically amplify minus strand strong stop DNA or LTR-LTR circular
25 junction sequence, respectively.   The positions of the PCR products are indicated.

Figure 4B Cells were transiently transfected with DNA of the MLV viral vector expressing luciferase (MLV-Luc), or
30 control vector expressing luciferase under CMV promoter (CMV-Luc).   Forty-eight hours after transfection, cells were lysed and luciferase activity was measured.

Figure 4C Analysis of viral RNA levels in infected cells.
Cells infected with undiluted Eco-Luc virus, and forty-
eight hours post infection total RNA, nuclear RNA and
cytoplasmic RNA fractions were isolated.   20 micrograms
5   of RNA from each fraction was resolved by electropheresis
in agarose gel and then transferred to a Nylon membrane.
The membrane was probed with P32-labeled luciferase DNA
and exposed to X-ray film (top panel).   The same membrane
was stipped and re-probed with 32P-labeled GAPDH DNA aand
10   exposed to X-ray film (middle panel).   To monitor the
integrity of the RNA samples, the gel was stained with
ethidium bromide (bottom panel) before transfer to the
membrane.   The relative RNA levels were quantitated by
Phosphoimager and plotted.   The positions of each RNA are
15   indicated.   E, Rat2-empty vector control cells; Z, Rat2-
NZAP-zeo cells.

Figure 4D Biological activity of various forms of rZAP.
Rat2 cells stably expressing the empty vector (black bar)
20   or NZAP-zeo (grey bar) were transiently transfected with
an empty vector DNA, a plasmid expressing the full-length
ZAP (pZAP-myc), or a plasmid expressing a fragment (NZAP-
myc).   The cells were then challeneged with MLV-Luc virus
and following forty-eight hour lysates, were assayed for
25   luciferase.   The full-length ZAP inhibited infection,
whicle the ZAP fragment relieved the inhibition caused by
NZAP-zeo.

Figure 5A   Amino acid sequence of the ZAP protein.
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Figure 5B   Nucleic acid sequence of ZAP cDNA.

Detailed Description of the Invention

Definitions

5   As   used   in   this   application,   except   as   otherwise
expressly provided herein, each of the following terms
shall have the meaning set forth below.

As used herein, "administering" shall mean delivering in
10   a manner which is effected or .performed using any of the
various methods and delivery systems known to those
skilled in the art.   Administering can be performed, for
example,   intravenously,   orally,   via   implant,
transmucosally,   transdermally,   intramuscularly,   or
15   subcutaneously.   "Administering" can. also be performed,
for example, once, a plurality of times, and/or over one
or more extended periods.

As   used   herein,   "agent"   shall   include,   without
20   limitation,   an   organic   compound,   a   nucleic   acid,   a
polypeptide,   a   lipid,   and   a   carbohydrate.   Agents
include, for example, agents which are known with respect
to structure and/or function, and those which are not
known with respect to structure or function.
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As used herein, "conditions permitting entry of the ZAP
protein   into   the   cell"   include,   for   example,
physiological conditions.

30   As used herein, "host cells" include, but are not limited
to, bacterial cells, yeast cells, fungal cells, insect
cells, and mammalian cells.   Mammalian cells · can be
transfected by methods well-known in the art such as

calcium phosphate precipitation, electroporation and microinjection.

As used herein, "increasing resistance" to a virus shall
5   mean inhibiting the replication of the virus in a cell infected therewith. In one embodiment, this inhibition is characterized by a reduction in mRNA encoding viral proteins.

10 As used herein, "mammalian cell" shall mean any mammalian cell. Mammalian cells include, without limitation, cells which are normal, abnormal and transformed, and are exemplified by neurons, epithelial cells, muscle cells, blood cells, immune cells, stem cells, osteocytes,
15 endothelial cells and blast cells.

As used herein, "nucleic acid" shall mean any nucleic acid molecule, including, without limitation, DNA, RNA and hybrids thereof. The nucleic acid bases that form
20 nucleic acid molecules can be the bases A, C, G, T and U, as well as derivatives thereof. Derivatives of these bases are well known in the art, and are exemplified in PCR Systems, Reagents and Consumables (Perkin Elmer Catalogue 1996-1997, Roche Molecular Systems, Inc.,
25 Branchburg, New Jersey, USA).

As used herein, "pharmaceutically acceptable carriers" are well known to those skilled in the art and include, but are not limited to, 0.01-0.1 M and preferably 0.05 M
30 phosphate buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers can be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil,

and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions and suspensions, including saline and buffered media. Parenteral vehicles include sodium

5   chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's and fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other

10   additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases, and the like.

As used herein, "protein" and "polypeptide" are used

15   equivalently, and each shall mean a polymer of amino acid residues. The amino acid residues can be naturally occurring or chemical analogues thereof. Polypeptides and proteins can also include modifications such as glycosylation, lipid attachment, sulfation,

20   hydroxylation, and ADP-ribosylation.

As used herein, "protein instability" shall mean the propensity, with respect to a protein, to undergo degradation or other modification adversely affecting the

25   function of the protein.

As used herein, "reporter sequence" shall mean a nucleotide sequence whose expression in a mammalian cell gives rise to a detectable signal.

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As used herein, RNA "corresponding" to a virus includes, without limitation, RNA normally found within the virus (as in the case of a retrovirus), and mRNA produced by a

cell infected with the virus using DNA from the virus as a template.

As used herein, "subject" shall mean any animal, such as
5   a non-human primate, mouse, rat, guinea pig, dog, cat, or rabbit.

As used herein, "vector" shall mean any nucleic acid vector known in the art. Such vectors include, but are
10   not limited to, plasmid vectors, cosmid vectors, and bacteriophage vectors.

As used herein, "virus" shall mean any of a large group of microscopic infective agents that are regarded either
15   as the smallest microorganisms or extremely complex molecules and are composed of a protein coat surrounding an RNA or DNA core of genetic material and are capable of growth and multiplication in living cells.

20   As used herein, "WWE" shall mean a globular protein domain in proteins involved in protein-protein interactions in ubiquitin and ADP-ribose conjugation systems.

25   As used herein, "ZAP" and "ZAP protein" are used synonymously, and each shall mean a mammalian protein which (a) comprises four CCCH-type zinc finger motifs, (b) binds to RNA corresponding to at least one type of virus ("target virus"), and (c) when present in a
30   mammalian cell infected with a target virus, binds to RNA corresponding to the target virus, so as to inhibit replication of the target virus in the cell.

As used herein, a ZAP protein "specific" for a virus
shall mean a ZAP protein known to cause the degradation
of RNA corresponding to that virus.

5   Embodiments of the Invention

This invention provides an isolated ZAP protein. In one
embodiment, the protein is a human protein. In a further
embodiment, the protein is a rat protein. In another
10   embodiment, the protein is a mouse protein. In yet
another embodiment, the protein comprises the amino acid
sequence set forth in SEQ ID NO:1

The instant protein can have deleted from it a region
15   which causes protein instability. In one embodiment, the
deleted region is the WWE region.

This invention further provides an isolated nucleic acid
which encodes a ZAP protein. In one embodiment, the
20   nucleic acid is DNA. In a further embodiment, the DNA is
cDNA. In another embodiment, the cDNA comprises the
nucleic acid sequence set forth in SEQ ID NO: 2. In yet
another embodiment, the DNA is genomic DNA. In yet
another embodiment, the nucleic acid is RNA.
25

In one embodiment, the nucleic acid encodes a human ZAP
protein. In yet another embodiment, the nucleic acid
encodes a rat ZAP protein. In yet another embodiment,
the nucleic acid encodes a mouse ZAP protein.
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The instant nucleic acid can encode a ZAP protein which
has deleted from it a region which causes protein
instability. In one embodiment, the deleted region is
the WWE region.

The instant nucleic acid can be labeled with a detectable
marker.   In one embodiment, the detectable marker is a
radioactive label, a calorimetric marker, a luminescent
5   marker or a fluorescent marker.

This invention further provides an expression vector
comprising a nucleic acid encoding a ZAP protein.   In one
embodiment, a host vector system comprises the expression
10   vector   and   a   suitable   host   cell.   In   a   further
embodiment, the host cell is a eukaryotic, bacterial,
insect or yeast cell.   In another embodiment, the host
cell is a mammalian cell.

15   This invention further provides a method for increasing
the amount of ZAP protein in a mammalian cell which
comprises contacting the cell with a ZAP protein under
conditions permitting entry of the ZAP protein into the
cell, so as to thereby increase the amount of ZAP protein
20   in the mammalian cell.

This invention further provides a method for increasing
the expression of ZAP protein in a mammalian cell which
comprises introducing into the cell an expression vector
25   comprising   a   nucleic   acid   sequence   encoding   a   ZAP
protein, so as to thereby increase ZAP protein expression
in the mammalian cell.

In   one   embodiment   of   the   instant   method,   the   method
30   comprises   the   step   of   detecting   the   increase   in   ZAP
protein expression by detecting a difference in   the
amount of ZAP-protein encoding mRNA in the mammalian cell
.before and after introduction of the expression vector
into the cell.

This invention further provides a method for increasing
resistance to a virus in a mammalian cell which comprises
contacting the cell with a ZAP protein specific for that
virus under conditions permitting entry of the ZAP
5   protein into the cell, so as to thereby increase
resistance to the virus in the subject.

In one embodiment of the instant method, the mammalian
cell is a human cell. In another embodiment, the virus
10   is an alpha virus. In yet another embodiment, the virus
is West Nile virus.

This invention further provides a method for increasing
resistance to a virus in a mammalian cell which comprises
15   introducing into the cell an expression vector comprising
a nucleic acid sequence encoding a ZAP protein specific
for that virus, so as to thereby increase resistance to
the virus in the mammalian cell.

In one embodiment of the instant method, the mammalian
20   cell is a human cell. In another embodiment method, the
virus is an alpha virus. In yet another embodiment, the
virus is West Nile virus.

25   This invention further provides a method for increasing
the amount of ZAP protein in a subject's cells which
comprises administering to the subject an amount of ZAP
protein effective to increase the amount of ZAP protein
in the subject's cells. In one embodiment of the instant
30   method, the subject is human.

This invention further provides a method for increasing
resistance to a virus in a subject which comprises
administering to the subject an amount of ZAP protein

specific for that virus effective to increase the amount
of ZAP protein in the subject's cells, so as to thereby
increase resistance to the virus in the subject.

5   In one embodiment of the instant method, the subject is
human.   In another embodiment, the virus is an alpha
virus.   In yet another embodiment, the virus is West Nile
virus.

10   This invention further provides a method for determining
whether an agent increases ZAP protein expression in a
mammalian cell which comprises: (a) contacting the cell
with the agent under conditions permitting ZAP protein
expression; (b) determining the resulting amount of ZAP
15   protein expression in the cell; and (c) comparing the
amount of expression determined in step (b) with the
amount of ZAP protein expression determined in the
absence of the agent, whereby a greater amount of ZAP
protein expression in the presence of the agent relative
20   to that in the absence of the agent indicates that the
agent increases ZAP protein expression in a mammalian
cell.

In one embodiment of the instant method, the method
25   comprises determining the resulting amount of ZAP protein
expression by determining the amount of ZAP protein-
encoding mRNA in the mammalian cell.   In another
embodiment of the instant method, the agent is a ZAP
protein having deleted from it a region which causes
30   protein instability.   In yet another embodiment of the
instant method, the deleted region is the WWE region.

This invention further provides a method for determining
whether an agent increases resistance to a virus in a

mammalian cell, which comprises: (a) contacting the agent
with a mammalian cell having introduced thereto an
expression vector comprising a nucleic acid sequence
corresponding to the virus operatively linked to a
5   reporter sequence whose expression in a mammalian cell
gives rise to a detectable signal, wherein . RNA
corresponding to the virus is known to be degraded by a
ZAP protein present in the cell; (b) determining the
amount of signal produced in the cell by the reporter
10   sequence after contact with the agent; and (c) comparing
the amount of signal determined in step (b) to that
produced in the absence of the agent, whereby the amount
of signal produced in the presence of the agent being
less than that produced in the absence of the agent
15   indicates that the agent increases resistance to the
virus in the cell.

In one embodiment of the instant method, the agent is a
ZAP protein having deleted from it a region which causes
20   protein instability. The deleted region can be, for
example, the WWE region. In a further embodiment of the
instant method, the reporter sequence is lacZ.   In
another embodiment, the virus is an alpha virus. In yet
another embodiment, the virus is West Nile Virus.
25

This invention further provides a composition comprising
a ZAP protein and a pharmaceutically acceptable carrier.

This invention further provides a composition comprising
30   an expression vector comprising a nucleic acid sequence
encoding a ZAP protein, and a pharmaceutically acceptable
carrier.

This invention further provides an article of manufacture comprising a packaging material having therein a ZAP protein, and a label indicating a use for the ZAP protein for increasing resistance to a virus in a subject.

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Finally, this invention provides an article of manufacture comprising a packaging material having therein an expression vector comprising a nucleic acid sequence encoding a ZAP protein, and a label indicating a 10 use for the expression vector for increasing resistance to a virus in a subject.

This invention is illustrated in the Experimental Details section which follows. This section is set forth to aid 15 in an understanding of the invention but is not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

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Experimental Details

Synopsis

5   Viral replication requires that the incoming virus
    successfully target a site of replication, express viral
    mRNAs and proteins, and assemble progeny virions. Cells
    have evolved several mechanisms by which they can inhibit
    viral replication. To identify novel viral inhibitors,
10 large mammalian cDNA libraries for any genes which could
    protect cells from infection by a genetically marked
    retrovirus were generated and screened. Virus resistant
    cells were selected from pools of transduced clones, and
    an active antiviral cDNA was recovered from one such
15 line. The gene encoded a CCCH-type zinc finger protein
    dubbed "ZAP". Expression of the gene in rat fibroblasts
    caused a profound and specific loss of the viral mRNAs
    from the cytoplasm without affecting the levels of the
    nuclear mRNAs.

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Methods and Discussion

    A library of expressed cDNAs was constructed in a
    retroviral vector, termed pBabe-HAZ. This vector was
25 constructed by making modifications to pBabe-puro. The
    EcoRI and NotI sites in pBabe-puro were sequentially
    removed by digestion, polishing of the ends by Klenow
    polymerase and ligation. The puromycin resistance gene
    was replaced by a zeocin resistance gene prepared by PCR
30 with various components built in the primers. The up
    stream   primer   (5'ATAAGCTTGCCACCATGGCTTSTCCSTSTGSTGTTC
    CAGATATGCTGAATTCGGCGGCCGCGCCAAGTTGACCAGTGC-3') contained
    the HindIII cloning site, kozak consensus sequence, ATG
    start codon, HA tag and ECORI/NotI linker sequences,

with HA tag fused to the zero gene. The downstream primer 5'ATATCGATTCAGTCCTGCTCCTCGGC-3') contained the ClaI cloning site. The Lox P sequence was inserted by annealing two oligonucleotides (5'CTAGATAACTTCGTATAATG

5 TATGCTATACGAAGTTAT-3') and (5'CTAGATAACTTCGTATAGCATACAT TATACGAAGTTAT-3') and ligating the product into the unique NheI site in the U3 region of the 3'LTR. To minimize the background of parental vector in the cDNA library, a 1-kb stuffer sequence was inserted between

10 the EcoRI and NotI sites to disrupt the HA-Zeo open reading frame. cDNAs were then used to replace the stuffer. (Fig. 1A). Randomly primed cDNAs from wild-type Rat2 fibroblasts were inserted into the vector under the control of a constitutive promoter, such that a

15 hemagglutinin (HA) epitope tag was fused at the 5' end, and a Zeocin resistance gene at the 3' end, encoding HA-orf-Zeo fusion proteins. RNA extracted from RAT2 cells with RNA extraction kit (Amersham- Pharmacia) following the manufacturer's instructions. cDNA was synthesized

20 from the mRNA and cDNA synthesis kits (Stratagene) following the manufacturer's instructions with the following modifications: a) NotI-oligo(dT) primer (Amersham-Pharmacia) was used to replace XhoI-oligo(dt); b) for each reaction, 15ug, instead of 5 mg of mRNA was

25 used as template to favor the synthesis of short cDNA fragments. The cDNA was cloned into pBabe-Haz digested with EcoRI and NotI and the reaction products were used to transform Electromax bacteria by electroporation. A LoxP sequence, the site recognized by the Cre

30 recombinase, was inserted into the U3 region of the 3' Long Terminal Repeat (LTR), which is duplicated during reverse transcription of the vector so that LoxP sited are present in both LTRs of the provirus after integration. These sites are positioned such that the

provirus can be excised from the genome by the Cre
recombinase. The complexity of the library was 2 x 10^7,
with inserts ranging in size from 0.2 kb to 3 kb.

5   A scheme was developed to identify antiviral genes in
the library (outlined in Fig. IB). Aliquots of the
library DNAs were used to transform 293T cells along
with DNAs encoding the Moloney MuLV Gag-Pol proteins and
the VSV G envelope protein, producing 20 pools of
10   pseudotyped transducing viruses.   To generate the
library of transducing viruses, a mixture of three DNAs,
the pBabe-HAZ-Rat2 cDNA library, the Gag-Pol gpt
plasmid, and the pMDG plasmid for the production of VSV-
G, was transiently introduced into 293T cells by calcium
15   phosphate-mediated   transformation.   The   culture
supernatant was collected 60 hours after transfection
and used to infect Rat2 cells.   While an antiviral gene
in the library might cause loss of the corresponding
transducing virus, it was hypothesized that the high and
20   transient expression in 293T cells would override its
activity. The transducing viruses were used to infect
thymidine   kinase-negative   (TK-)   Rat2   cells,   and
recipient clones were selected by culture in zeocin. Each
clone in the pooled cells overexpressed a single member
25   of the cDNA library.

To identify any genes in the pooled cells that conferred
retrovirus   resistance,   we   made   use   of   a   powerful
selection for virus-resistant cells (14). The pools of
30   transduced TK- Rat2 cells were challenged by repeated
infection   with   both   ecotropic   and   amphotropic
retroviruses expressing the TK gene from the viral
promoter. The bulk of the virus-sensitive cells, now
having become TK positive, were then killed by growth in

the toxic thymidine analogue trifluorothymidine (TFT),
and rare TK-negative clones were recovered as candidate
virus-resistant cells. Out of a total of 5×10^5
transduced   lines   put   through   the   selection,

5   approximately 200 TFT-resistant clones were isolated.
Retests of each of the clones showed that one, line
L1D3, was dramatically resistant to virus infection. L1D3
cells were approximately 30-fold less sensitive than the
parent cells to viruses carrying a luciferase reporter

10   (Eco-Luc virus).

To confirm that the cDNA insert in the L1D3 line was
responsible   for   the   resistance,   the   cells   were
transfected   with   a   construct   expressing   the   Cre

15   recombinase to induce the excision of the provirus at the
LoxP   sites   and   the   loss   of   the   cDNA.   Five   stable
transfectants   were   analyzed   for   their   resistance   to
infection by Eco-Luc virus and for the presence or loss
of the cDNA by PCR amplification of the genomic DNA (Fig.

20   2A).   To   generate   Ecotropic   MLV-luciferase   virus,
retroviral   vector   pSRαL-Luc   was   stably   introduced   into
GP+E86 producer cells by cotransformation with pSV2-Neo.
Approximately 100 G418-resistant clones were individually
tested for release of Eco-Luc virus.   The clone that

25   produced the highest titer of virus was explained and
used for subsequent experiments.   Those clones from which
the cDNA was excised were all susceptible to infection,
while   the   clones   that   retained   the   cDNA   were   all
resistant. These experiments confirmed that the cDNA was

30   indeed necessary for the virus resistance of the L1D3
line.

The cDNA insert in L1D3 was recovered from the genomic
DNA by PCR amplification and cloned. To recover the cDNA

insert from the L1D3 cell line, 1mg of genomic DNA was
used as a template in a 50 ml PCR reaction with the
Expand High Fidelity PCR kit under the following
conditions: 10 cycles of 94oC for 15 seconds, 50oC for 30
5   seconds, 72oC for 60 seconds each cycle, followed by 20
cycles of 94oC for 15 seconds, 55oC for 30 seconds, 72oC
for 60+5 seconds each cycle.   The sense primer was
5'GCTTATCCATATGATGTTCCAGATT-3', and the antisense primer
was CZAP-ap-AP (5'ATATAGGCGGCCGCCCTCTGGACCTCTTCTCTTC-3').
10   To confirm that the cDNA was sufficient to induce virus
resistance, the cDNA was recloned into the pBabe-HAZ
vector and then reintroduced into naive Rat2 cells.
Cells expressing the cDNA were again 30-fold resistant to
the Eco-Luc virus as compared to the parental cells or
15   cells carrying the empty vector (Fig. 2B). Thus, the
expression of the cDNA was sufficient to establish viral
resistance.

The DNA sequence of the insert revealed a single long
20   open reading frame of 254 codons fused to the zeocin
resistance gene at its 3' end (Fig. 3A). The insert
contained a long 5' untranslated region (UTR) and the
ORF was not fused to HA at the 5' end; translation of
the HA sequence terminated in the UTR and expression of
25   the protein required translation initiation at an AUG
codon at the start of the ORF, in the context of a good
match to a Kozak consensus start site. Searches of the
nucleic acid databases with the coding region revealed
two mouse EST clones with highly similar sequences
30   (mEST995 and mEST896). These two sequences were identical
to each other except for differences at their very 3'
ends, which probably arise by alternative splicing
events. The sequence of mEST995 was used to design PCR
primers, and the full-length sequence of the rat cDNA

was amplified and cloned. The C-terminal portion of ZAP was cloned from a Rat2 cell cDNA library by PCR using sense primer CZAP-SP (5'GAGCTCTCTGGGCTTAACC-3') and antisense primer CZAP-AP (5'ATATAGGCGGCCGCCCTCTGGAC

5 CTCTTCTCTTC-3'). The sense primer lies upstream from an internal NheI site in NZAP; the antisense primer introduces a NotI site (bolded) immediately downstream from the coding sequence to facilitate its cloning into the myc-tagged expression vector. PCR was conducted with

10 Expand High Fidelity PCR kit (Roche) under the following conditions: 10 cycles of 94oC for 15 seconds, 50oC for 30 seconds, 72oC for 120 seconds each cycle, followed by 20 cycles of 94oC for 15 seconds, 55oC for 30 seconds and 72oC for 120+5 seconds each cycle. The PCR product was

15 digested with NheI and NotI and then cloned into pCDNA4/TO2-NZAP-myc. The sequence of the complete cDNA contained 789 codons (sequence deposited in GenBank, accession # pending); the initial cDNA corresponded perfectly to the aminoterminal one-third of the

20 sequence. The predicted amino acid sequences of the rat protein and of the similar mouse proteins contained a cluster of four unusual CCCH-type zinc fingers, previously found in only a few RNA-binding proteins (Fig 3B). The gene was dubbed rZAP, for rat Zinc-finger

25 Antiviral Protein, and the initial antiviral N-terminal fusion construct was named NZAP-zeo.

The resistance to virus transduction could be effected at many stages of infection. To determine the position of

30 the block in the retrovirus life cycle, Rat2 cells expressing NZAP-zeo or the empty vector as control were acutely infected with Eco-Luc virus and the synthesis of viral DNA was examined by PCR. Comparable levels of minus strand strong stop DNA were synthesized in both

lines, suggesting no block to entry or initiation of
reverse transcription (Fig. 4A). Similar experiments
with appropriate primers showed no defects in plus
strand DNA synthesis. To test for nuclear entry of the

5   viral DNA, the cells were infected with a similar vector,
Eco-GFP, but with a higher titer, and circular viral DNAs
were analyzed by PCR amplification of the LTR-LTR
junction. Comparable levels of the circular forms were
detected (Fig. 4A), suggesting no block to nuclear entry.

10   To test for defects in viral gene expression, the
retroviral Luc reporter DNA was directly introduced into
the cells by lipid-mediated transformation, bypassing
all the early stages of infection. The cells expressing
NZAP-zeo expressed 30-fold lower luceriferase than the

15   controls, the same decrease seen after retroviral
infection (Fig. 4B). These results suggest that NZAP-zeo
does not affect early events but rather acts to inhibit
retroviral gene expression from the viral DNA. The
effect was specific for retroviral expression, since

20   expression of a control reporter construct via mRNAs
lacking viral sequences was only reduced three fold in
the NZAP-zeo line (Fig. 4B).

To determine the mechanism of the resistance to virus
25   gene expression, viral mRNA levels in cells expressing
NZAP-zeo or the empty vector were measured. After
infection with Eco-Luc virus, total cellular RNAs were
prepared, and in addition, cells were fractionated and
nuclear and cytoplasmic RNAs were isolated. Cells of

30   each line were infected with freshly collected Eco-Luc
virus for at least five hours. Two days after infection
the cells were trypsinized and collected for RNA
preparation. Approximately 20% of the cells were used to
extract total RNA with RNA extraction kit (Qiagen). The

rest of the cells were used to extract cytoplasmic RNA with the the RNA extraction kit (Qiagen) following manufacturer's instructions. The nuclear pellet from cytoplasmic RNA extraction was washed twice with the

5 lysis buffer and the nuclear RNA was then extracted as for total RNA. The RNAs were separated by gel electrophoresis, blotted, and probed with 32P-labelled luciferase sequences (Fig. 4C). The whole-cell preparations showed a modest reduction in level of the

10 viral RNA in the NZAP-zeo cells as compared to the control cells. The fractionated RNAs, however, showed a dramatic result. The levels of the viral RNA in the nuclear fraction were virtually identical in the NZAP-zeo and control lines, suggesting that there was no

15 significant effect on transcription initiation or elongation. But the cytoplasmic viral RNA was almost completely abolished in the NZAP-zeo cells, while high levels were found in the controls. Quantitation of longer exposures indicated a 30-fold reduction in the

20 levels of the cytoplasmic RNA relative to the controls (Fig. 4C). The levels of rRNA and of a housekeeping mRNA, GAPDH, were unaffected. These results suggest that the NZAP-zeo protein specifically eliminated the cytoplasmic fraction of the viral RNA.

25

The antiviral construct as originally isolated contained only the 5' one third of the complete rZAP coding region, fused to the zeocin resistance gene. The complete rZAP protein could have similar activity as the fusion

30 protein; alternatively, the full protein could normally have a positive activity that is antagonized by a dominant negative activity of the fragment. To test the function of the full-length protein, the complete ZAP ORF was cloned into an expression plasmid under the

control of the CMV promoter, tagged with a myc epitope
at the carboxyterminus, forming pZAP-myc. The pZAP-myc
was used to transform Rat2 cells expressing the empty
vector control, and also those expressing NZAP-zeo, and
5   the effects on Eco-Luc transduction were measured as
before. The full-length protein induced a dramatic
inhibition of viral vector expression on its own (Fig.
4D). The inhibition was even greater when rZAP and NZAP-
zeo were expressed together. These results suggest that
10   the full-length rZAP also acts to negatively regulate
viral transcripts.

Other constructs were tested to look for forms of ZAP
that would interfere with the antiviral activity of the
15   wild-type protein. The 5' portion of the gene present in
NZAP-zeo was excised from the pBabe-HAZ vector and
expressed without the zeo fusion partner and with a myc
epitope tag. The NZAP fragment was excised from pBabe-
NZAP-Zeo with EcoRI-NotI and cloned into pCDNA4/TO2-myc-
20   HisB (Invitrogen) to generate a myc-tagged NZAP. The
fragment reproducibly caused a small increase in the
level of luciferase detected after infection by Eco-Luc
virus (Fig. 4D). Moreover, this fragment almost
completely suppressed the inhibition in cells containing
25   NZAP-zeo or rZAP, restoring normal luciferase
expression. Thus, this fragment antagonized the normal
ZAP activity.

The results above show that direct selections for virus-
30   resistant cells (14) can be used to identify new genes
with potent antiviral activity. Key aspects to the
selection were the use of highly infectible parental
cells; transfer of a large cDNA library to the cells;
repeated   and   saturating   infection   with   a

counterselectable virus; and the efficient killing of virus-sensitive cells. The gene recovered here, rZAP, is sufficient on its own to induce an antiviral state with no apparent affect on cell viability or physiology. The
5   wild-type rZAP, as well as the truncated NZAP-zeo fusion protein, causes a profound inhibition of expression of reporter genes carried by retroviral vectors, acting at the level of the cytoplasmic viral RNA.

10 ZAP prevents the accumulation of cytoplasmic viral RNA because of the presence of the cluster of four unusual CCCH-type zinc fingers suggests that ZAP may interact directly with the viral RNA. These fingers are found in a small family of RNA binding proteins; the best-known
15 member of the family is tristetraprolin (TTP), a protein which negatively regulates the levels of TNF-oc (24) and GM-CSF mRNAs (25). TTP binds AU-rich sequences in the 3' UTR of the TNF-a mRNA (24,26) and recruits the exosome to degrade the mRNA (27); it acts in opposition to the
20 binding of HuR, another RNA-binding protein which stabilizes its target. rZAP may act in a similar way at sequences found in viral RNAs, and perhaps also in specific cellular mRNAs. Consistent with this notion, preliminary tests of ZAP mutants suggest that all of the
25 finger motifs are crucial for its activity (data not shown). However, there is little sequence similarity to TTP outside the fingers, and the distinctive parts of the molecule may carry out other functions than the induction of RNA degradation.

30
The normal function of rZAP may be to regulate one or more specific cellular mRNAs. However, like PKR, RNase L, and the MX proteins, rZAP may be an example of a gene whose primary function will prove to be inhibiting viral

gene expression and inducing an innate immunity to viral
infection. The full range of viruses restricted by rZAP
is not yet known. Preliminary results, however, suggest
that the gene may have an extended antiviral activity;
5   for example, rZAP can potently block replication of
Sindbis virus in Rat2 cells (M. MacDonald, personal
communication). Activation of expression of the
endogenous gene could ultimately help induce immunity
and protect individuals from disease caused by viral
10   infections.

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